MOLECULAR AND CELLULAR BIOLOGY, Nov. 2009, p. 5952–5962 Vol. 29, No. 21
0270-7306/09/$12.00 doi:10.1128/MCB.00585-09

Phosphoinositide (3,4,5)-Triphosphate Binding to Phosphoinositide-Dependent Kinase 1 Regulates a Protein Kinase B/Akt Signaling Threshold That Dictates T-Cell Migration, Not Proliferation▽

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Received 5 May 2009/Returned for modification 17 June 2009/Accepted 13 August 2009

The present study explored the consequences of phosphoinositide (3,4,5)-triphosphate [PI(3,4,5)P_3] binding to the pleckstrin homology (PH) domain of the serine/threonine kinase 3-phosphoinositide-dependent kinase 1 (PDK1). The salient finding is that PDK1 directly transduces the PI(3,4,5)P_3 signaling that determines T-cell trafficking programs but not T-cell growth and proliferation. The integrity of the PDK1 PH domain thus is not required for PDK1 catalytic activity or to support cell survival and the proliferation of thymic and peripheral T cells. However, a PDK1 mutant that cannot bind PI(3,4,5)P_3 cannot trigger the signals that terminate the expression of the transcription factor KLF2 in activated T cells and cannot switch the chemokine and adhesion receptor profile of naïve T cells to the profile of effector T cells. The PDK1 PH domain also is required for the maximal activation of Akt/protein kinase B (PKB) and for the maximal phosphorylation and inactivation of Foxo family transcription factors in T cells. PI(3,4,5)P_3 binding to PDK1 and the strength of PKB activity thus can dictate the nature of the T-cell response. Low levels of PKB activity can be sufficient for T-cell proliferation but insufficient to initiate the migratory program of effector T cells.

Signal transduction pathways that are important in thymocytes and peripheral T lymphocytes include those regulated by class I phosphoinositide 3-kinases (PI3Ks) that phosphorylate the 3′-OH position of the inositol ring of phosphatidylinositol (4, 5)-biphosphate to produce the lipid product phosphoinositide (3,4,5)-triphosphate [PI(3,4,5)P_3]. This lipid binds to the pleckstrin homology (PH) domains of proteins and controls the activity and subcellular localization of a diverse array of signal transduction molecules that are fundamental for the growth, proliferation, and differentiation of T lymphocytes (14, 20, 22). PI3K signaling is critical for early T-cell development and controls the survival and proliferation of T-cell progenitors (49). In mature peripheral T cells, PI3K signaling regulates proliferation and differentiation and, in particular, controls nutrient uptake, protein synthesis, and the cell growth of immune-activated T cells (13, 36). PI(3,4,5)P_3 signaling via mTOR (mammalian target of rapamycin) and Foxo family transcription factors also controls lymphocyte trafficking between the blood lymphoid organs and peripheral T cells by determining the repertoire of adhesion and chemokine receptors expressed by T lymphocytes (16, 27, 45).

One immediate consequence of increasing cellular PI(3,4,5)P_3 is the activation of the serine/threonine kinase protein kinase B (PKB) or Akt. The loss of PI3Ks or the deletion of PKB isoforms causes similar metabolic and survival defects in T-cell progenitors, indicating that PKB is a key effector of PI(3,4,5)P_3 signaling pathways (18, 25, 32). A rate-limiting step for PKB activation is the phosphorylation of threonine 308 (T308) within the PKB catalytic domain. This crucial event is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and is initiated by increases in cellular levels of PI(3,4,5)P_3. It is known that the loss of PDK1 in T-cell progenitors phenocopies the loss of PI3Ks or PKB isoforms and causes a block in T-cell development at the pre-T-cell stage (23). However, the role of PDK1 in the thymus is not confined to the activation of PKB in response to the generation of PI(3,4,5)P_3 but also extends to the activation of other AGC kinases (26). Hence, the replacement of wild-type (WT) PDK1 with a PDK1-L155E mutant that supports full PKB activation is not sufficient to restore normal thymocyte development (26). The replacement of leucine (L) 155 in PDK1 with glutamate (E) disrupts the integrity of an important PDK1 domain, termed the PIF binding pocket (12). This domain is not required for PKB phosphorylation but is necessary for PDK1 to interact with carboxy-terminal hydrophobic motifs in other members of the AGC kinase family, such as p70 ribosomal S6 kinases (S6Ks) and p90 ribosomal S6 kinase (RSK) (12). The role of PDK1 in T-cell progenitors thus reflects that this kinase is essential to phosphorylate multiple members of the AGC kinase family (26).

One confusing issue about PDK1 is whether it is a direct mediator of PI(3,4,5)P_3 signal transduction. PDK1 does have a

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▽ Published ahead of print on 24 August 2009.

PH domain that binds PI(3,4,5)P_3 with high affinity (15, 28). The PDK1 phosphorylation of PKB also is PI(3,4,5)P_3 dependent (1). These data originally were interpreted to mean that PDK1 activity was PI3K dependent (hence the name 3-phosphoinositide-dependent protein kinase-1). However, subsequent work has shown that the catalytic activity of PDK1 is constitutively high (1). Moreover, the binding of PI(3,4,5)P_3 to its PH domain is not required for PDK1 catalytic activity but promotes the localization of the enzyme to the plasma membrane, where it can colocalize with PKB (15). Rather, the PI(3,4,5)P_3 dependence of PKB activation reflects that PI(3,4,5)P_3 binding to the PKB PH domain causes a conformational change that allows PDK1 to phosphorylate T308 within the PKB catalytic domain and activate the kinase (9, 34).

In T lymphocytes, PI(3,4,5)P_3 plays a role in localizing PDK1 to the T-cell immune synapse (35). It also has been reported that increases in intracellular PI(3,4,5)P_3 levels induced by agonistic CD28 antibodies bind to PDK1, recruit PDK1 to the plasma membrane, and trigger PDK1-induced phosphorylation and the activation of protein kinase Cθ (PKCθ) (29). Hence, the deletion of PDK1 in peripheral CD4 T cells is associated with an inability of the cells to produce interleukin-2 (IL-2) (29). In this context, the impact of deleting PDK1 phenocopies the impact of inhibiting PI3Ks (36). Accordingly, it has been argued that PDK1 is an important mediator of PI3K/PI(3,4,5)P_3 signal transduction in T cells and functions to coordinate T-cell receptor (TCR) and CD28 signal transduction. However, the contribution of PI(3,4,5)P_3 binding to the PDK1 PH domain for PDK1 function during T-cell development and in peripheral T cells has not been tested directly. In this context, recent studies have found that mutations in the PDK1 PH domain that block PI(3,4,5)P_3 binding do not compromise PDK1 function during embryogenesis (7). Hence, mice with deletions in both PDK1 alleles do not survive embryogenesis beyond embryonic day 9.5, whereas mice homozygous for a knock-in mutant of PDK1 incapable of binding PI(3,4,5)P_3 (PDK1 K465E) are viable. Moreover, PDK1 K465E mice are fertile and appear phenotypically normal, albeit significantly smaller, than normal mice and are prone to insulin resistance. Strikingly, the loss of PI(3,4,5)P_3 binding to the PDK1 PH domain in tissues from PDK1 K465E mice did strongly reduce PKB phosphorylation. However, the submaximal levels of PKB activity that can be supported by the PDK1 K465E mutant clearly were sufficient for the cellular functions of PKB during embryogenesis and in adult somatic tissues (7). In the present study, we have used PDK1 K465E mice to explore the role of PI(3,4,5)P_3 binding to PDK1 in T cells. These studies reveal that the integrity of the PDK1 PH domain is required for the maximal activation of PKB in T cells and is required for the maximal phosphorylation and inactivation of Foxo family transcription factors in T cells. However, PI(3,4,5)P_3 binding to PDK1 was not required for the survival, differentiation, or proliferation of thymocytes or peripheral T cells. One critical function for PI(3,4,5)P_3 binding to PDK1 was identified in T cells: namely, to redirect the trafficking of immune-activated effector T cells. The present study thus establishes that PDK1 controls a critical subset of PI(3,4,5)P_3-mediated signal transduction pathways in T cells but also has substantial and important PI(3,4,5)P_3-independent activity.

MATERIALS AND METHODS

Mice. Mice carrying a knock-in mutation, a substitution of lysine for glutamic acid at residue 465 in the PH domain of PDK1 (PDK1^{K465E}), were generated by homologous recombination and embryo transfer as previously described (7). Mice homozygous for this mutation were bred from matings of heterozygous pairs. To generate PDK1^{K465E} TCR transgenic mice, PH domain mutant mice were crossed with P14 TCR transgenic mice. The P14 TCR comprises a $V_\alpha 2V_\beta 8.1$ complex that recognizes a peptide, gp33-41 (GP33) (KAVYNFATM), of the lymphocytic choriomeningitis virus (LCMV) in the context of the H-2D^b major histocompatibility loci (40). All mice were bred and maintained in the Wellcome Trust Biocenter at the University of Dundee in compliance with United Kingdom Home Office Animals (Scientific Procedures) Act 1986 guidelines.

Cell culture. Spleens were removed from 2- to 6-month-old mice, mashed in cell strainers, and disaggregated, and red blood cells were lysed and suspended in RPMI 1640 medium containing L-glutamine (Invitrogen) with 10% heat-inactivated fetal calf serum (Gibco), penicillin-streptomycin (Gibco), and 50 μM β-mercaptoethanol (Sigma) and were activated for 48 h with either 1 μM soluble LCMV TCR-specific peptide gp33-41 (KAVYNFATM) or 0.5 μg/ml anti-CD3 antibody (2C11). Following activation, cells were washed to remove peptide or 2C11 antibody and cultured in media supplemented with cytokines, with or without inhibitors, as indicated in the text. Recombinant human IL-2 (Proleukin) and recombinant human IL-15 (Peprotech) were used at a final concentration of 20 ng/ml, and cells were cultured at a density of approximately 0.25×10^6/ml. The PI3K inhibitor LY294002 (Promega) was used at a final concentration of 10 μM, the mTOR inhibitor rapamycin (Calbiochem) was used at a final concentration of 20 nM, and the PKB inhibitor AktI 1/2 (Calbiochem) was used at a final concentration of 1 μM.

Flow cytometry. Unless otherwise stated, antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or biotin were from BD Pharmingen; PE-Cy5.5-conjugated antibodies were from Caltag. Cells were stained for the surface expression of the following antigens (clones are in parentheses): CD4 (RM4-5), CD8 (53-6.7), CD25 (7D4 or PC61), CD71 (C2), CD98 (RL388), CD62L (MEL-14), CD69 (H1.2F3), Thy1.2 (53-2.1), TCR-β (H57-597), TCR-γ/δ (GL3), B220 (RA3-6B2), Vα2 (B20.1), and $V_\beta 8.1$ (F23.1). CD4 (MCD0418) and CD8 (MCD0818) both were from Caltag; granzyme B (16G6) was from eBioscience. CD4 and CD8 double-negative (DN) subsets were gated by the lineage exclusion of all CD4, CD8 double-positive and single-positive (DP and SP, respectively) cells and TCR-γ/δ. DN3s and DN4s were further defined as CD25$^+$ CD44$^-$ and CD25$^-$ CD44$^-$ thymocytes, respectively. Mature SP thymocytes were defined as Thy-1$^+$ TCRβ^{hi} and positive for CD4 or CD8 expression.

For CCR7 staining, cells were labeled with a fusion protein of mouse CCL19 and a human Fcγ fragment (catalog no. 14-1972) and detected using PE-conjugated anti-human Fcγ (catalog no. 12-4998; both from eBiosciences). Where required, Fc receptors were blocked with mouse Fc block (CD16/CD32; FcgIII/II receptor; 2.4G2) (BD Pharmingen). Cells were stained with saturating concentrations of antibody in accordance with the manufacturer's instructions and were washed and resuspended in RPMI 1640–0.5% fetal bovine serum (FBS) prior to acquisition. Live cells were gated according to their forward scatter and side scatter. Data were acquired on either a FACSCalibur (Becton Dickinson) or an LSR1 (Becton Dickinson) flow cytometer and analyzed using FlowJo software (Treestar).

Phospho-S6 ribosomal protein intracellular staining. Cells were washed, fixed in 0.5% paraformaldeyde at 4°C, washed in phosphate-buffered saline (PBS), and permeabilized with 90% methanol (30 min, −20°C). After being washed again, cells were blocked with 0.5% bovine serum albumin in PBS (10 min, room temperature [RT]) and then incubated with primary anti-phospho-S6 ribosomal protein Ser 235/236 (Cell Signaling Technologies) for 30 min at RT. Cells were washed and then incubated with FITC- or PE-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch) for 30 min at RT in the dark. Samples were washed and data acquired on a FACSCalibur flow cytometer. As a positive control, maximal phospho-S6 staining was achieved with the pharmacological stimulation of cells using phorbol 12,13-dibutyrate (PdBu) (Calbiochem) (20 ng/ml, 30 min, 37°C). As a negative control, rapamycin (Calbiochem) (20 nM, 30 min, 37°C) was used to inhibit S6 phosphorylation.

Proliferation assays. Proliferation was assessed either by the incorporation of tritiated thymidine or by cell counting. To assess proliferation in response to TCR induction, splenocytes from TCR transgenic mice were cultured with 2C11 or GP33, with or without IL-2, for 48, 72, or 96 h at 1×10^5 cells/well in 96-well, flat-bottom plates at 37°C in a 5% CO_2 humidified incubator. At each time point during the last 4 h of culture, 1 μCi (0.037 mBq) tritiated [^{3}H]thymidine (Am-

ersham Biosciences) was added to each well. Cells were harvested using vacuum aspiration onto glass matrix filters. Incorporated radioactivity was quantified with a β-microplated scintillation counter. To assess proliferation in response to IL-2, splenocytes activated for 48 h with 2C11 or GP33 and then cultured for an additional 72 h with IL-2 were diluted to 0.25×10^6/ml and cultured for an additional 48 h with titrated amounts of IL-2. At 24 and 48 h, samples were taken for cell counts and phenotyping. Accurate counts were obtained using beads (Caltag) and flow cytometry in accordance with the manufacturer's recommendations. Viability was assessed on the basis of forward scatter, side scatter, and the exclusion of the viability marker 7-amino-actinomycin D (7AAD) (Sigma).

Western blot analysis. Biochemistry was assessed using standard Western blotting protocols. Briefly, cell lysates (20 million cells/ml) were prepared on ice using NP-40 lysis buffer (50 mM HEPES [pH 7.4], 75 mM NaCl, 1% NP-40, 10 mM sodium fluoride, 10 mM iodoacetimide, 1 mM EDTA, 40 mM β-glycerophosphate, protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 100 μM sodium orthovanadate) and then centrifuged at $1,600 \times g$ for 20 min at 4°C. Protein samples (0.25 to 0.5 million T-cell equivalents per track) were separated by electrophoresis through sodium dodecyl sulfate–4 to 12% polyacrylamide gels (Invitrogen), transferred to nitrocellulose membranes, and blocked with 5% milk fat in PBS-Tween 20. Blots were probed with antibodies recognizing the phosphorylated proteins PKB Thr308 or Ser473, GSK-3α/β Ser21/Ser9, Foxo1/o3a Thr24/Thr32, p70 S6 kinase Thr421/Ser424, S6 ribosomal protein Ser235/Ser236 (Cell Signaling Technologies), and RSK Ser227 (Upstate) and the nonphosphorylated (total) proteins PKB GSK-3α/β, p70 S6 kinase, S6 ribosomal protein, RSK (Cell Signaling Technologies), PDK1 (Millipore), Foxo1, and Foxo3 (in-house antibodies raised in sheep against full-length human Foxo1 and the N terminus of human Foxo3a, both of which cross-react with the mouse proteins).

Plasmid constructs. PDK1WT-green fluorescent protein (GFP), Foxo3aWT-GFP, and the Foxo3 triple mutant T32A/S252A/S314A (Foxo3AAA-GFP) constructs were obtained from the College of Life Sciences Cloning Service, University of Dundee. The retroviral vector pBMN-Z (Addgene) was digested with HindIII and NotI to remove *lacZ*; a 0.7-kb insert containing enhanced GFP (EGFP) (NCBI AAB02574.1) was excised from EGFP-N1 (Clontech) using HindIII and NotI and inserted into pBMN in place of *lacZ* (pBMN-GFP). WT PDK1 was excised from pCR-BluntII-TOPO-PDK1WT using EcoRI and NotI and inserted into the digested pBMN vector (pPDK1WT-GFP). WT Foxo3a(NCBI NP_062714.1) was amplified from FANTOM clone B930059F01 (the use of which has been licensed from The Institute of Physical and Chemical Research [RIKEN]) using Phusion polymerase (Finnzymes). The PCR product was cloned into pSC-b (Stratagene) and sequenced to completion. The insert was excised using EcoRI and inserted upstream of the EGFP into the pBMN vector to generate pFoxo3a-GFP. To generate the Foxo3a triple mutant, the Foxo3a insert was excised from the pSC-b Foxo3a construct using BamHI and HindIII and inserted into EGFP-N1. Point mutations were created using a QuikChange mutagenesis kit (Stratagene). The mutated insert was amplified using Phusion polymerase, digested with EcoRI, and inserted upstream of EGFP in the pBMN vector (pFoxo3AAA-GFP). All constructs were verified by sequencing.

Retrovirus production. Phoenix ecotropic packaging cells (Stanford University) were transfected with 5 to 10 μg of plasmid (pBMN-GFP, pPDK1WT-GFP, pFoxo3aWT-GFP, or pFoxo3AAA-GFP) using a standard calcium phosphate transfection protocol. Approximately 12 to 18 h after transfection, the medium was discarded and fresh medium added to the dishes. After an additional 24 h of incubation (37°C, 5% CO_2), retroviral supernatants were collected and spun briefly (1,500 rpm, 5 min) to sediment and remove packaging cells. The supernatant was transferred to fresh tubes, and viral particles were concentrated by high-speed centrifugation ($20,000 \times g$ for 4 h). Following centrifugation, supernatant was discarded, and concentrated viral particles were resuspended in 1 ml medium, snap-frozen, and stored at −80°C.

Retroviral transduction of primary T cells. Splenic T cells were activated for 12 to 24 h with either 2C11 or cognate peptide, as appropriate. Cells were counted, and 10^6 cells (at 2×10^6 cells/ml) were transferred into each well of a 12-well plate. Freshly thawed retrovirus supernatant (1 ml) and polybrene (Sigma) at a final concentration of 10 μg/ml were mixed and added to each well of cells. Plates were spun ($650 \times g$, 45 to 60 min), and then 1 ml of medium containing IL-2 (20 ng/ml) was added to each well and plates were incubated (37°C, 5% CO_2). The following day, cells were centrifuged to remove polybrene and activating agent, resuspended in fresh medium containing IL-2, and incubated as before. Cells were assessed for infection efficiency at 48 h using flow cytometry to detect GFP^+ cells.

Real-time PCR. Cell lysates were prepared and RNA extracted using the RNeasy RNA purification Mini kit (Qiagen) according to the manufacturer's protocol, including the on-column digestion of genomic DNA. Reverse transcription-PCR was performed using an iScript cDNA synthesis kit (Bio-Rad) in accord with the specified protocol. Real-time PCR was done on an iCycler (Bio-Rad) using iQ SYBR green-based detection (Bio-Rad). Primers for housekeeping genes and genes of interest were the following: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5′-CAT GGC CTT CCG TGT TCC TA-3′; GAPDH reverse, 5′-CCT GCT TCA CCA CCT TCT TGA T-3′; hypoxanthine phosphoribosyltransferase (HPRT) forward, 5′-TGA TCA GTC AAC GGG GGA CA-3′; HPRT reverse, 5′-TTC GAG AGG TCC TTT TCA CCA-3′; 18S forward, 5′-ATC AGA TAC CGT CGT AGT TCC G-3′; 18S reverse, 5′-TCC GTC AAT TCC TTT AAG TTT CAG C-3′; CD62L forward, 5′-ACG GGC CCC AGT GTC AGT ATG TG-3′; CD62L reverse, 5′-TGA GAA ATG CCA GCC CCG AGA A-3′; KLF2 forward, 5′-TGT GAG AAA TGC CTT TGA GTT TAC TG-3′; KLF2 reverse, 5′-CCC TTA TAG AAA TAC AAT CGG TCA TAG TC-3′; CCR7 forward, 5′-CAG GCT TCC TGT GTG ATT TCT ACA-3′; CCR7 reverse, 5′-ACC ACC AGC ACG TTT TTC CT-3′; sphingosine 1 phosphate receptor 1 ($S1P_1$) forward, 5′-GTG TAG ACC CAG AGT CCT GCG-3′; $S1P_1$ reverse, 5′-AGC TTT TCC TTG GCT GGA GAG-3′.

Adoptive transfer. T cells from either $PDK1^{WT}$ or $PDK1^{K465E}$ TCR transgenic mice were activated with GP33 for 48 h, washed, and then cultured for an additional 72 h in IL-2 (20 ng/ml final concentration). Cells were labeled (10 to 20 min, 37°C water bath) with either 5-(-6)-{[(4-chloromethyl)benzoyl]amino} tetramethylrhodamine (CMTMR; CellTracker Orange) (Molecular Probes Invitrogen) or carboxyfluoroscein diactetae succinimidyl diester (CFSE) (Molecular Probes Invitrogen), washed, and mixed in a 1:1 ratio in sterile PBS. Cell suspensions (10^7 cells in 100 μl) were injected into the tail vein of C57BL/6 mice, and 24 h later mice were killed and tissues taken for the quantification of CMTMR- or CFSE-labeled T cells by flow cytometry.

Statistical analyses. Statistical analyses were performed using GraphPad Prism 4.00 for Macintosh (GraphPad Software). A nonparametric Mann-Whitney test was used where the number of experiments performed was not sufficient to prove normal distribution. For comparisons of gene expression between WT and PH domain mutant animals, a Student's *t* test was used with the theoretical mean of the WT samples set to 1. Where a time course of gene expression was compared between inhibitor-treated and untreated samples, a two-way repeated measures analysis of variance was used.

RESULTS

$PI(3,4,5)P_3$ binding to PDK1 is not essential for thymocyte development. Mice homozygous for PDK1 alleles with a K465E mutation are viable and fertile, although they have a selective signaling defect that impairs the phosphorylation and activation of PKB in all tissues (7). Consequently, PDK1 K465E mice are approximately 30% smaller than WT littermate controls (mean weights ± standard deviations [SD] for males: $PDK1^{WT/WT}$, 26.0 ± 3.5 g, $n = 25$; $PDK1^{K465E/K465E}$, 20.3 ± 3.9 g, $n = 14$) (mean weights ± standard deviations for females: $PDK1^{WT/WT}$, 21.5 ± 4.1 g, $n = 22$; $PDK1^{K465E/K465E}$, 16.3 ± 3.9 g, $n = 17$). Several studies have demonstrated the importance of $PI(3,4,5)P_3$ and PDK1 signaling pathways in thymocyte development (23, 25, 32, 49). For example, in mice the deletion of PDK1, or the combined deletion of the PI3K p110γ and δ catalytic subunits, in T-cell progenitors blocks thymocyte development at the pre-T cell stage prior to the expression of the major histocompatibility complex (MHC) receptors CD4 and CD8 (23, 49). Such mice thus have a very small thymus comprised almost entirely of $CD4^-/CD8^-$ DN cells and effectively are devoid of mature thymic and peripheral T cells (26, 49). In contrast, thymi from $PDK1^{K465E/K465E}$ mice have the normal frequency of CD4/CD8 DP and CD4 or CD8 SP thymocytes (Fig. 1A). These mice also have a normal frequency of thymocytes expressing high levels of the mature α/β T-cell antigen receptor complex, a marker of mature thymic SP thymocytes (Fig. 1B).

In normal T-cell progenitors, PDK1-mediated phosphorylation and the activation of PKB controls the activation of the 70-kDa ribosomal S6 kinase 1 (S6K1), which then phosphory-

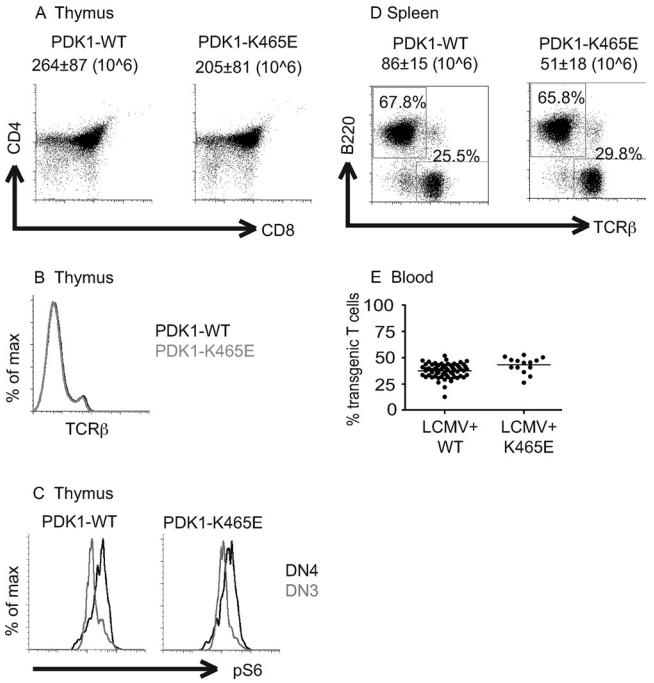

FIG. 1. PI(3,4,5)P_3 binding to PDK1 is not essential for thymocyte development, positive selection, or the generation of peripheral T cells. (A) Expression of CD4/CD8 DP and SP subsets in thymi from PDK1^{WT} or PDK1^{K465E} mice. Total thymocytes are indicated over each dot plot as means ± SD ($n = 14$; $P < 0.02$). (B) Expression of TCR-ß on thymocytes from PDK1^{WT} and PDK1^{K465E} mice. (C) Ribosomal S6 phosphorylation (S235/236) in DN4 cells compared to that of DN3 cells in PDK1^{WT} and PDK1^{K465E} thymocytes. (D) Proportions of T and B cells in spleens from PDK1^{WT} and PDK1^{K465E}. Total splenocytes are indicated over each dot plot as means ± SD ($n = 11$; $P < 0.0001$). (E) PDK1^{K465E} mice were backcrossed to P14 TCR transgenic mice. Data show the frequency of $V_\alpha2V_\beta8.1$ transgenic TCR expressing CD8-positive T cells in blood from P14 TCR transgenic PDK1^{WT} and PDK1^{K465E} mice. Each dot represents a mouse.

lates S6 ribosomal subunits (24). Hence, in a normal thymus, the transition of pre-T cells from the DN3 to the DN4 subsets is accompanied by the increased phosphorylation of ribosomal S6 subunits (24). This response is lost following PDK1 deletion (24) but is normal in PDK1$^{K465E/K465E}$ DN thymocytes (Fig. 1C). In DN4 T-cell progenitors, the PDK1-mediated activation of PKB is essential for the expression of the nutrient receptors CD71 (transferrin receptor) and CD98 (a subunit of the L-amino acid transporter) and controls cell growth (cell mass) (26). PDK1$^{K465E/K465E}$ DN4 thymocytes are of normal size (large blastoid cells) and express normal levels of the nutrient receptors CD71 and CD98 (data not shown). Thus, there is no functional loss of PDK1/PKB/S6K1 signaling in T-cell progenitors expressing the PDK1 K465E mutation.

A striking consequence of PDK1 deletion in T-cell progenitors is reduced thymic cellularity (reduced by 90 to 95% compared to that of normal mice) and a failure to produce peripheral T cells (23). Thymic cellularity was lower than normal in PDK1$^{K465E/K465E}$ mice, but the level of reduction was consistent with their overall reduced size and body weight. Importantly, PDK1$^{K465E/K465E}$ mice have a normal frequency of T cells in the spleen (Fig. 1D), lymph node, and peripheral blood (data not shown). These data suggest that the integrity of the PDK1 PH domain is not required for thymocyte development. To further probe this issue, PDK1$^{K465E/K465E}$ mice were backcrossed to P14 TCR transgenic mice. P14 TCR transgenic T cells express a $V_\alpha2V_\beta8.1$ TCR that drives the positive selection of thymocytes to the CD8 lineage in mice expressing the H-2D^b MHC. Figure 1E shows that the frequencies of $V_\alpha2V_\beta8.1$ TCR transgenic T cells in WT and PDK1$^{K465E/K465E}$ mice are indistinguishable. PI(3,4,5)P_3 binding to PDK1 thus is not needed for positive selection during thymus development.

PI(3,4,5)P_3 binding to PDK1 is not required for peripheral T-cell activation and proliferation or the differentiation of cytotoxic T cells. To assess whether PI(3,4,5)P_3 binding to PDK1 was required to support the proliferation of mature T cells, we compared the proliferative responses of WT and PDK1$^{K465E/K465E}$ T cells triggered via the TCR with cognate antigen/MHC. The $V_\alpha2V_\beta8.1$ TCR expressed on P14 TCR transgenic T cells can be triggered with a peptide of LCMV, glycoprotein gp33-41 (KAVYNFATM), which is presented by the MHC class I (MHC-I) molecule H-2D^b. The ability of P14 TCR transgenic T cells to proliferate in response to peptide stimulation is dependent on the production of PI(3,4,5)P_3. Figure 2A (left) thus shows that the PI3K inhibitor, IC87114, which selectively inhibits the p110δ PI3K catalytic subunit (42), prevents TCR-induced DNA synthesis. However, P14 TCR transgenic T cells that express the PDK1 K465E mutation show a normal proliferative response to both suboptimal and saturating concentrations of the gp33-41 peptide (Fig. 2A, right). The PDK1 PH domain thus does not mediate PI(3,4,5)P_3 signaling for T-cell proliferation.

The triggering of antigen receptors induces the expression of IL-2 receptors and induces a state of IL-2 responsiveness in peripheral T cells. Moreover, antigen-primed T cells cultured in IL-2 for 3 to 7 days proliferate and differentiate to generate effector cytotoxic T cells (CTL). The data in Fig. 2B show that the ability of IL-2 to promote cell proliferation (left panel) and viability (right panel) of WT CTL compared to that of PDK1$^{K465E/K465E}$ CTL is indistinguishable. Activated PDK1$^{K465E/K465E}$ T cells express CD69 and CD25, the alpha subunit of the IL-2 receptor, at normal levels (Fig. 2C). In response to TCR stimulation, PDK1$^{K465E/K465E}$ T cells from lymph node (Fig. 2D) and spleen (data not shown) produce IL-2 at levels similar to those of WT T cells. Effector CTL express granzyme B and upregulate the expression of nutrient receptors such as CD71 (transferrin receptor) and CD98 (L-amino acid transporter) (Fig. 2E, upper).

Activated PDK1$^{K465E/K465E}$ CTLs generated by the culture of TCR-activated T cells in IL-2 also express comparable levels of the cytolytic effector molecule granzyme B (Fig. 2E, bottom) and can kill antigen-primed target cells (data not shown). In effector T cells, the levels of expression of CD71 (transferrin receptor) and CD98 (L-amino acid transporter) are PI3K dependent and determined by cellular levels of PI(3,4,5)P_3 (13). PI3K activity also controls T-cell growth (13, 41). PDK1$^{K465E/K465E}$ effector T cells express normal levels of CD71 and CD98 (Fig. 2E, lower middle and right, respectively) and are of normal size (data not shown). PI(3,4,5)P_3 binding to PDK1 thus is not needed for nutrient receptor expression or the growth of T cells.

PI(3,4,5)P_3 binding to PDK1 controls T-cell trafficking. One consistent difference between antigen-induced WT and

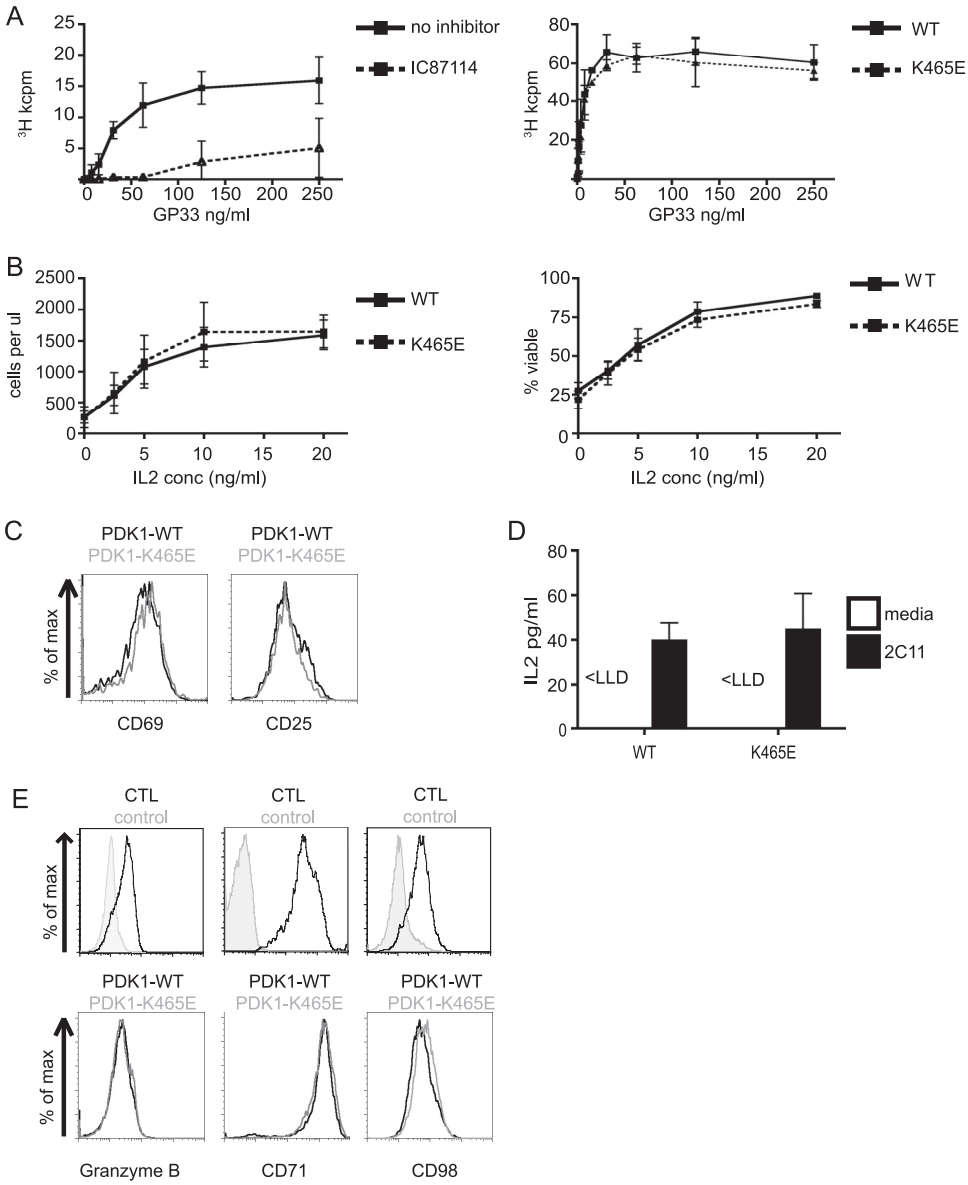

FIG. 2. PI(3,4,5)P_3 binding to PDK1 is not essential for the proliferation and viability of mature T cells. (A) The graph on the left shows that the proliferation of T cells in response to peptide stimulation is dependent on PI(3,4,5)P_3 production. Data show tritiated [^{3}H]thymidine incorporation into P14 TCR transgenic T cells stimulated for 48 h with LCMV gp33 peptide in the presence or absence of the PI3K inhibitor IC87114. The graph on the right shows the dose response of [^{3}H]thymidine incorporation into P14 LCMV splenic T cells primed with LCMV gp33-41 peptide for 48 h. (B) Splenic T cells were activated with 2C11 for 48 h and cultured for an additional 2 days with IL-2, at which point cells were washed and subcultured into the indicated concentration of IL-2 or medium alone. Data show the proliferation (left) and viability (right) of spleen-derived CTL. Flow cytometry was used to assess cell concentration (conc) and viability at 48 h of treatment. (C) Cell surface expression of CD69 (left) and CD25 (right) on splenic T cells activated with 2C11 for 24 to 48 h. PDK1^{WT}, black line; PDK1^{K465E}, gray line. (D) IL-2 production from lymph node T cells activated for 18 h in the presence or absence of 2C11. Bars indicate means ± standard errors of the means (n = 3). <LLD, below the lower level of detection. (E) The top row shows the expression of granzyme B (left), CD71 (middle), and CD98 (right) in effector CTL (black line). Background staining (gray line) for granzyme B staining in IL-15-maintained CD8 memory T cells is shown. Naive CD8 T cells were used for background staining for CD98 and CD71. The bottom row shows the expression of granzyme B (left), CD71 (middle), and CD98 (right) on effector CTL cultured in IL-2 (20 ng/ml). PDK1^{WT}, black line; PDK1^{K465E}, gray line.

PDK1$^{K465E/K465E}$ effector CTL is that WT effector cells express low levels of the adhesion molecule CD62L (L-selectin), whereas the PDK1$^{K465E/K465E}$ effector cells express high levels of CD62L (Fig. 3A, left). The downregulation of CD62L expression by effector T cells is a normal consequence of effector T-cell differentiation and occurs because of the transcriptional silencing of *CD62L* (11, 45). Thus, there are low levels of CD62L mRNA in WT effector CTL (Fig. 3A, right). In contrast, there are high levels of CD62L mRNA in P14PDK1$^{K465E/K465E}$ CTL (Fig. 3A, right). Previous studies have shown that the downregulation of *CD62L* gene transcription in CTL is sustained by high levels of PI(3,4,5)P_3

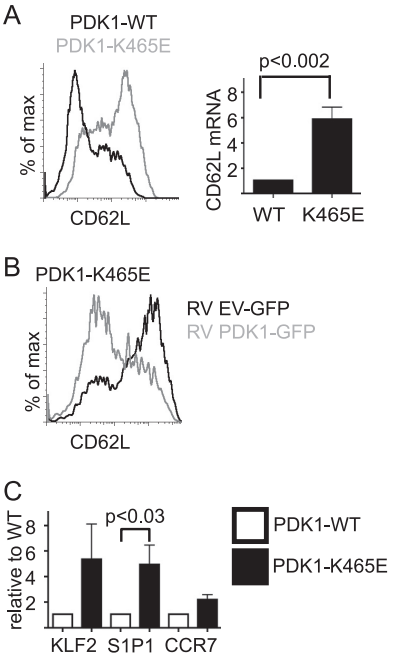

FIG. 3. PI(3,4,5)P3 binding to PDK1 controls the expression of lymph node homing receptors. (A) Data show the surface expression (left) and gene expression (right) of CD62L (L-selectin) in activated splenic T cells cultured for 2 to 3 days in IL-2 (20 ng/ml). Gene expression bars indicate means ± standard errors of the means; $n = 6$. (B) Splenic T cells from PDK1^{K465E} mice were activated overnight with 2C11 and then infected with virus expressing either empty vector-GFP (RV EV-GFP; black lines) or WT PDK1 (RV PDK1-GFP; gray lines) and thereafter cultured with IL-2 (20 ng/ml). Data show the surface expression of CD62L 5 days after infection and are representative of two separate experiments. (C) Gene expression of KLF2 (left) and the KLF2 targets $S1P_1$ (center) and CCR7 (right) in effector CTL. P14 LCMV CD8$^+$ T cells were activated with LCMV gp33-41 peptide for 2 days and then cultured with IL-2 for an additional 2 to 3 days. Gene expression is relative to that in WT CTL (set at 1). Bars indicate means ± standard errors of the means; $n = 5$.

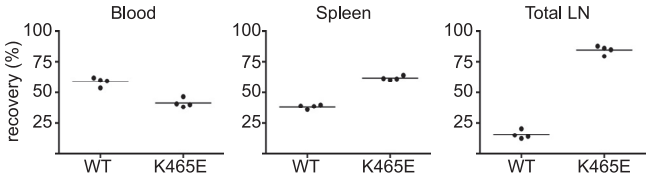

FIG. 4. PI(3,4,5)P3 binding to PDK1 controls T-cell trafficking. Data show the recovery of fluorescently labeled PDK1^{WT} and PDK1^{K465E} CTL adoptively transferred into the WT host. P14 CD8$^+$ cells activated for 2 days with gp33-41 and then cultured for 3 days with IL-2 (20 ng/nl) were labeled with CFSE or CMTMR, respectively, and mixed at a 1:1 ratio before being injected into C57BL/6 host mice. Values indicate the recovery of PDK1^{WT} or PDK1^{K465E} cells as a percentage of total transferred cells recovered from the blood, spleen, or lymph nodes (LN) (data pooled from separate analyses of mesenteric, axial, and inguinal nodes) at 24 h after transfer. Each dot represents a mouse, and data are from two separate experiments.

signaling (45). The data in Fig. 3 thus are consistent with a model in which PDK1 acts as a direct effector of PI(3,4,5)P_3 to silence CD62L expression. If this hypothesis is correct, then the reexpression of WT PDK1 in PDK1$^{K465E/K465E}$ effector CTL should cause the loss of CD62L expression. Accordingly, we used retroviruses to reexpress WT PDK1 in PDK1$^{K465E/K465E}$ T cells. Figure 3B shows CD62L expression in PDK1$^{K465E/K465E}$ effector T cells transduced with virus expressing either GFP alone (EV-GFP) or GFP-tagged WT PDK1 (PDK1-GFP). Strikingly, the reexpression of WT PDK1 in PDK1$^{K465E/K465E}$ effector T cells caused the downregulation of CD62L to the levels seen in WT T cells. In contrast, PDK1$^{K465E/K465E}$ T cells transduced with GFP alone maintained high levels of CD62L.

CD62L/L-selectin is expressed at high levels in naive and memory T cells and controls the adhesion of these cells to the endothelium of high endothelial venules (HEV) and, hence, is essential for lymphocyte transmigration from the blood into secondary lymphoid tissue such as lymph nodes (2, 30, 50). The loss of CD62L expression following immune activation thus is part of the program that redirects the trafficking of effector T cells away from lymphoid tissue toward non-lymphoid peripheral tissue and sites of inflammation (31, 46). One other change in effector T cells is the loss of the expression of the chemokine receptor CCR7, which directs the migration of T cells into secondary lymphoid organs and also controls their motility and positioning within lymphoid tissues. Immune-activated T cells also downregulate the expression of $S1P_1$, which controls lymphocyte egress from secondary lymphoid organs (43). The coordinated loss of CD62L, CCR7, and $S1P_1$ occurs because their expression is controlled by a common transcription factor, KLF2, which is expressed at high levels in naive T cells and downregulated by immune activation (3, 8, 10, 45). We therefore examined whether the differences in *CD62L* transcription between WT and PDK1$^{K465E/K465E}$ effector T cells reflects differences in KLF2 expression and is accompanied by differences in the expression of *CCR7* and *S1P$_1$*. Real-time PCR analysis revealed that KLF2 mRNA levels were low in WT effector CTL but increased in PDK1$^{K465E/K465E}$ effector CTL (Fig. 3C). Moreover, PDK1$^{K465E/K465E}$ effector T cells showed increased $S1P_1$ and CCR7 mRNA expression compared to that of WT effector CTL (Fig. 3C).

Differences between WT and PDK1$^{K465E/K465E}$ CTL in terms of the expression of KLF2, CD62L, CCR7, and $S1P_1$ indicates that the PDK1$^{K465E/K465E}$ CTL have not switched their trafficking behavior to that of an effector CTL and have retained the capacity to home to lymphoid tissues. To test this possibility in vivo, adoptive transfer experiments were performed that compared the ability of WT effector CTL and PDK1$^{K465E/K465E}$ CTLs to home to secondary lymphoid tissues. In these experiments, WT or PDK1$^{K465E/K465E}$ P14 T cells were activated via TCR triggering with gp33-41 peptide for 2 days, followed by 3 days of culture in IL-2. Cells then were labeled with CFSE or CMTMR, mixed at a ratio of 1:1, and transferred into C57BL/6 hosts. After 24 h, the mice were sacrificed and tissue was analyzed for the presence of the transferred cells. Strikingly, PDK1$^{K465E/K465E}$ CTLs, but not WT CTL, retained the capacity to home to secondary lymphoid organs and, hence, accumulated in lymph nodes and spleen rather than disseminating to peripheral tissues (Fig. 4). These results reveal that PI(3,4,5)P_3 binding to the PDK1 PH domain is required for the normal programming of CTL trafficking.

PI(3,4,5)P_3 binding to PDK1 is required for optimal PKB activation and Foxo phosphorylation. The expression of KLF2

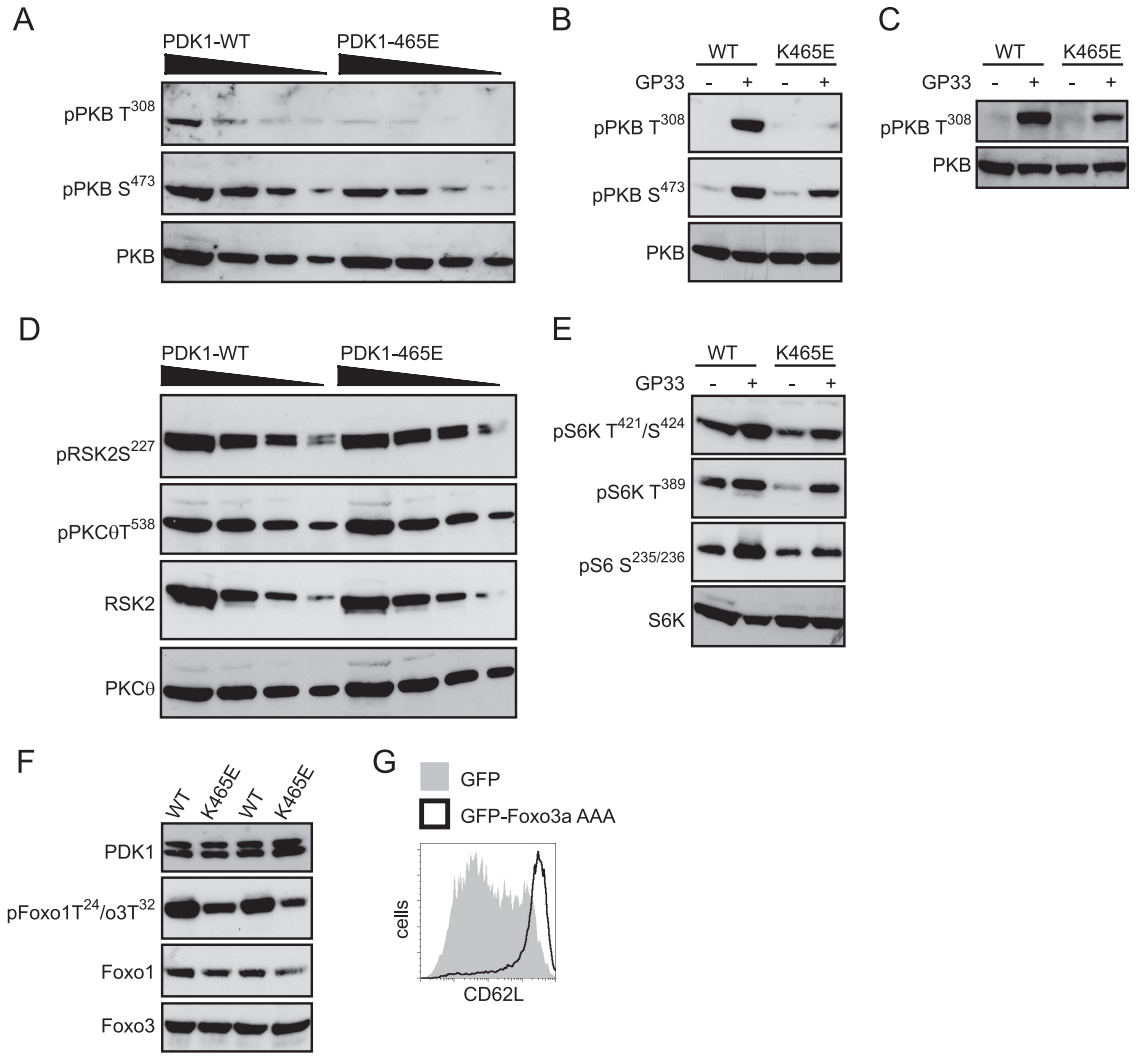

FIG. 5. PI(3,4,5)P3 binding to PDK1 is required for optimal PKB activation. (A) Data show PKB phosphorylation in PDK1^{WT} and PDK1^{K465E} splenic T cells activated with 2C11 for 48 h and then cultured in IL-2 for an additional 3 days. Triangles indicate cell titration. (B) PKB phosphorylation in PDK1^{WT} and PDK1^{K465E} P14 LCMV CD8^{+} T cells activated with cognate peptide (gp33-41) and cultured in IL-2 (20 ng/ml) to generate CTL and then retriggered with peptide for 15 min. (C) PKB phosphorylation in primary PDK1^{WT} and PDK1^{K465E} P14 LCMV CD8^{+} T cells activated with cognate peptide (gp33-41) for 15 min. (D) RSK2 S227 and PKCθ T538 phosphorylation in PDK1^{WT} and PDK1^{K465E} splenic T cells activated with 2C11 for 48 h and then cultured in IL-2 for an additional 3 days. Triangles indicate cell titration. (E) Data show the phosphorylation of S6 kinase and S6 proteins in PDK1^{WT} and PDK1^{K465E} P14 LCMV CTL retriggered with cognate peptide (gp33-41) for 15 min. (F) Phosphorylation of Foxo proteins in PDK1^{WT} and PDK1^{K465E} splenic T cells activated with 2C11 for 48 h and then cultured in IL-2 for an additional 3 days. Data are from two WT and two mutant mice. (G) Splenic T cells were activated overnight with 2C11 and then infected with virus expressing either GFP or a GFP-tagged Foxo3a mutant with alanine substitutions at its PKB substrate sites T32, S252, and S314 (GFP-Foxo3aAAA). The surface expression of CD62L was assessed 2 days after infection.

and CD62L is controlled by Foxo family transcription factors such as Foxo1 and Foxo3a (16, 17, 27, 37). In naïve T cells, Foxo1 and Foxo3a reside in the nucleus (16) and drive high levels of *KLF2* and *CD62L* transcription (16). In immune-activated T cells the stimulation of PI3K activates PKB, which phosphorylates Foxo1 and Foxo3a, resulting in their nuclear exclusion and the termination of Foxo-mediated gene transcription (16, 17). The high levels of KLF2 and CD62L gene transcription in PDK1$^{K465E/K465E}$ CTLs therefore could be explained by the defective activation of PKB and a failure of these cells to phosphorylate and inactivate Foxo transcription factors. The experiment shown in Fig. 5A addresses this issue and compares the phosphorylation and activity of PKB in stimulated PDK1$^{WT/WT}$ and PDK1$^{K465E/K465E}$ effector CTL cultured in IL-2. The data show there was a reduced phosphorylation of PKB on its PDK1 substrate site T308 in PDK1$^{K465E/K465E}$ T cells compared to that of PDK1$^{WT/WT}$ cells, and PKB phosphorylation on the PDK2 site serine 473 (S473) was normal. The triggering of the TCR can induce further PKB T308 phosphorylation in IL-2-maintained CTL. The data (Fig. 5B) show that this antigen receptor-induced response also was impaired in PDK1$^{K465E/K465E}$ T cells. There also was reduced PKB T308 phosphorylation in TCR-triggered naïve PDK1$^{K465E/K465E}$ T cells compared to that of control cells (Fig. 5C). It recently has

been suggested that PI(3,4,5)P_3 binding stimulates PDK1 catalytic activity in vitro (38). We therefore assessed whether the in vivo catalytic activity of PDK1 in T lymphocytes is directly dependent on PI(3,4,5)P_3 binding. To address this issue, we examined PDK1$^{K465E/K465E}$ effector CTL for the phosphorylation of the PDK1 substrate S227 in the RSK2 catalytic domain. Figure 5D shows the normal phosphorylation of RSK2 S227 in PDK1$^{K465E/K465E}$ cells. Hence, in T lymphocytes, PI(3,4,5)P_3 binding to PDK1 is required for optimal PKB phosphorylation but is not globally required for PDK1 catalytic function. One other proposed PDK1 substrate in T lymphocytes is T538 in PKCθ, and phosphorylation on this site also was normal in PDK1$^{K465E/K465E}$ T cells (Fig. 5D).

The phosphorylation of PKB on T308 in its catalytic domain is rate limiting for PKB activity, but it is difficult to extrapolate directly the impact of the reduced PKB T308 phosphorylation on the in situ activity of PKB and the subsequent phosphorylation of PKB substrates. The results in Fig. 5E address this question and show that IL-2-generated CTL have reduced mTOR activity as assessed by the reduced basal and peptide-stimulated phosphorylation of S6K1 on the mTOR sites T421/S424 and T389. This defect reduced S6K1 activity, as judged by the lower levels of phosphorylated ribosomal S6 subunits in PDK1$^{K465E/K465E}$ T cells. In the context of these experiments, it is important to note that these reductions were not as severe as those seen in PDK1 null T cells, which show a complete loss of S6K1, S6, and Foxo phosphorylation and also lose RSK2 S227 phosphorylation (unpublished data). Furthermore, PDK1$^{K465E/K465E}$ T cells have reduced the phosphorylation of the Foxo1/3a transcription factors on the PKB substrate sites T24 and T32 (Fig. 5F). The biochemical analyses in Fig. 5 thus indicate that PIP3 binding to the PDK1 PH domain is required for the optimal activation of PKB in T cells but is not required for PDK1 to phosphorylate other substrates, such as RSK2.

One consequence of the suboptimal activation of PKB is a reduction in the phosphorylation of Foxo family members, which would favor the retention of Foxo transcriptional activity in activated PDK1$^{K465E/K465E}$ T cells and possibly explain the high levels of expression of KLF2 and its targets, such as CD62L. To test this hypothesis, it was important to assess whether the PKB-mediated phosphorylation of Foxo family members explains the downregulation of KLF2 and CD62L in normal immune activated effector CTL. We first assessed whether the restoration of Foxo transcriptional activity in WT activated T cells containing high levels of active PKB could restore CD62L expression. In these experiments, we examined whether the expression of a Foxo3a mutant with alanine substitutions at its PKB substrate sequences T32, S252, and S314 (Foxo3aAAA) could restore the expression of CD62L in WT immune-activated T cells. This mutant of Foxo3a cannot be phosphorylated and inactivated by PKB and should restore Foxo transcriptional function in cells expressing active PKB. Figure 5G shows that antigen receptor-activated T cells expressing low levels of CD62L on the surface can regain the expression of CD62L if infected with the Foxo3aAAA mutant. These data confirm that the expression of *CD62L* is regulated by Foxo transcription factors.

To assess whether differences in PKB activity directly modulate the expression of KLF2 and CD62L, a series of experiments with an inhibitor of PKB, Akti-1/2, were performed.

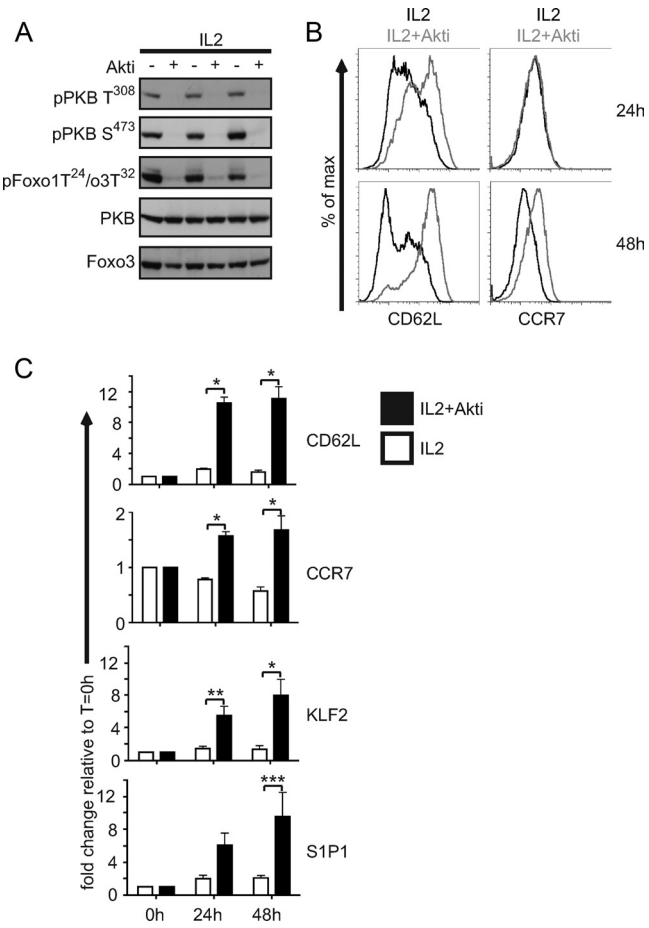

FIG. 6. PKB activity modulates the expression of trafficking molecules in T cells. (A) Regulation of PKB and Foxo phosphorylation in P14 LCMV CD8$^+$ cells activated for 2 days with gp33-41, maintained in IL-2 for an additional 3 days, and then left untreated or treated with the PKB inhibitor Akti-1/2 (1 μM) for 48 h. (B) Surface expression of CD62L (left) and CCR7 (right) on P14 LCMV CD8$^+$ cells cultured as described for panel A and then treated with or without the PKB inhibitor Akti-1/2 for 24 (upper) and 48 h (lower). Representative histograms of three separate experiments. (C) Gene expression in cells cultured as described for panel A. Bars indicate means ± standard errors of the means; $n = 3$. *, $P < 0.001$; **, $P < 0.05$; ***, $P < 0.01$.

Akti-1/2 is a highly selective allosteric inhibitor of PKB that prevents the conformational change that occurs when the PKB PH domain binds PI(3,4,5)P_3, thus inhibiting the PDK1-mediated phosphorylations that are essential for PKB activation (6). Thus, as shown in Fig. 6A, the addition of Akti-1/2 to WT CTL maintained in IL-2 caused a loss of PKB T308 and S473 phosphorylation and a resultant loss of PKB activity, as judged by the accompanying decrease in the phosphorylation of Foxo (Fig. 6A). The loss of PKB phosphorylation at T308 and S473 was apparent within 15 min of treatment with inhibitor (data not shown). When effector CTL were treated with Akti-1/2 for 24 to 48 h, they showed a striking increase in the surface expression of CD62L and CCR7 (Fig. 6B, left and right, respectively). This was caused by increased *CD62L* and *CCR7* mRNA expression (Fig. 6C, first and second graphs, respectively). The expression of *CD62L* and *CCR7* is controlled by the transcription factor KLF2, and in this context, Fig. 6C

(third graph) shows that treatment with the Akti-1/2 inhibitor resulted in increased expression of *KLF2* and the KLF2 target $S1P_1$ (Fig. 6C, fourth graph) in effector CTL.

DISCUSSION

The present study has explored the consequences of $PI(3,4,5)P_3$ binding to the PH domain of the serine/threonine kinase PDK1 for T-cell development and peripheral T-cell function. The salient findings are that the integrity of the PDK1 PH domain is required for the maximal activation of PKB in T cells and is required for the maximal phosphorylation and inactivation of Foxo family transcription factors in T cells. The impaired PKB activation caused by the loss of a functional PDK1 PH domain did not affect T-cell development in the thymus and also had no effect on the antigen receptor or cytokine triggered proliferation of peripheral T cells. These data reveal that low levels of PKB activation are sufficient to support T-cell proliferation. However, $PI(3,4,5)P_3$ binding to the PDK1 PH domain was required to redirect the trafficking of naïve T cells from the blood/secondary lymphoid tissue circuit. PDK1 thus acts as a direct mediator of the $PI(3,4,5)P_3$ signals that control lymphocyte migration but does not mediate the $PI(3,4,5)P_3$ signals that control T-cell growth and proliferation during T-cell development.

The present data show that there was normal phosphorylation of RSK2 on its PDK1 substrate sequence S227 in $PDK1^{K465E/K465E}$ T cells, which is unequivocal evidence that $PI(3,4,5)P_3$ binding is not essential for the catalytic function of PDK1. This conclusion was reinforced by in vitro kinase assays that found no difference in the catalytic activity of the WT compared to that of the K465E mutant of PDK1 (7). The confusion about the role of $PI(3,4,5)P_3$ in PDK1 activation arises because the ability of PDK1 to phosphorylate and activate PKB is tightly regulated by cell-extrinsic stimuli and dependent on increases in cellular $PI(3,4,5)P_3$ levels. However, structural studies have shown that the $PI(3,4,5)P_3$ dependence of PKB activation reflects that $PI(3,4,5)P_3$ binding to the PKB PH domain causes a conformational change that allows PDK1 to phosphorylate T308 within the PKB catalytic domain and activate the kinase (34). The reduction of PKB T308 phosphorylation in T cells expressing PDK1 K465E alleles thus reflects that $PI(3,4,5)P_3$ binding colocalizes PDK1 and PKB to promote the PDK1-mediated phosphorylation of the PKB catalytic domain. $PI(3,4,5)P_3$ thus has a scaffolding function in T cells to assemble and optimize PDK1/PKB interactions. This scaffolding role for $PI(3,4,5)P_3$ is relevant for PKB but not other substrates that scaffold with PDK1 via a protein interaction domain known as the PIF motif (12). In this context, it has been proposed that $PI(3,4,5)P_3$ control of PDK1 localization and activity is important for the phosphorylation of T538 in PKCθ (29). There has been some controversy about whether the phosphorylation of this T loop site in PKCθ is constitutive or regulated by immune activation and PI3K activity. The present data offer some resolution to this debate and show that mutations of the PDK1 PH domain that prevent PDK1/$PI(3,4,5)P_3$ binding and cause the loss of PKB T308 phosphorylation are permissive for PKCθ T538 phosphorylation. One key role for the T loop phosphorylation of PKCs is to stabilize protein expression such that defects in T loop phosphorylation cause a reduction in PKC protein levels (4, 5). Accordingly, the normality of PKCθ expression in $PDK1^{K465E/K465E}$ T cells is further evidence that PKCθ T loop phosphorylation is normal. Moreover, PKCθ null T cells have severe defects in the ability to produce cytokines or proliferate in response to immune stimulation (33, 47); the normality of these responses in $PDK1^{K465E/K465E}$ T cells also is strong evidence that these cells have normal PKCθ function. $PI(3,4,5)P_3$ binding to PDK1 thus is not required for the phosphorylation or function of PKCθ in T cells. This does not refute the model that PDK1 coordinates TCR/CD28 signal transduction but refines it further by excluding $PI(3,4,5)P_3$ binding to PDK1 as an important mechanism for PKCθ regulation (Fig. 7).

T-cell development in the thymus and the immune activation of primary T cells is dependent on PKB. Hence, it was interesting that the reduced levels of PKB activity in $PDK1^{K465E/K465E}$ T cells were sufficient to mediate cell survival and proliferation during thymus development and sufficient to support the immune activation of T cells. It was equally interesting that the low levels of PKB activity in $PDK1^{K465E/K465E}$ effector T cells were not sufficient to downregulate the expression of the transcription factor KLF2 or to switch the chemokine and adhesion receptor profile of naïve T cells to the profile of effector T cells. These data reveal that PKB does not function as a simple on/off switch in T cells; rather, $PI(3,4,5)P_3$ binding to PDK1 and the strength of PKB activity dictates the nature of the T-cell immune response. In this context, there are interesting parallels between the $PDK1^{K465E/K465E}$ effector T cells described herein and effector T cells that lack a functional PI3K p110δ catalytic domain. These p110δ-deficient T cells also can proliferate but fail to downregulate KLF2, CD62L, etc. (45). Low levels of PI3K and PKB activity thus can be sufficient for T-cell growth and proliferation but are not sufficient to initiate the migratory program of effector T cells.

The observation that $PI(3,4,5)P_3$ binding to PDK1 controls T-cell migration is particularly important, because understanding the molecular mechanisms that change the migratory pattern of T cells following immune activation is fundamental to understanding how to control T-cell immune responses. Naïve T lymphocytes constantly recirculate via the blood and lymphatics to secondary lymphoid organs. In contrast, effector T lymphocytes lose the capacity to home to secondary lymphoid tissues and migrate to a greater extent to peripheral tissues and sites of inflammation to mediate effector function. The changes in the migratory patterns of effector T cells are mediated by changes in the expression of chemokine receptors and adhesion molecules. For example, naïve T cells express CCR7 and follow a chemotactic gradient formed by the chemokines CCL19 and CCL21 from blood across HEV into secondary lymphoid tissue such as lymph nodes. CD62L/L-selectin binds to ligands in the HEV and mediates the capture of cells on the HEV, a necessary step for T-cell entry into lymph nodes. Effector T cells normally downregulate the expression of CCR7 and CD62L as part of the program of changes that redirect their trafficking away from secondary lymphoid tissue to peripheral tissues (19, 21, 48, 50). Immune-activated T cells also downregulate the expression of the $S1P_1$ that controls lymphocyte egress from secondary lymphoid organs (39, 43). The coordinated loss of CD62L, CCR7, and $S1P_1$ occurs because their expression is controlled by a common transcription fac-

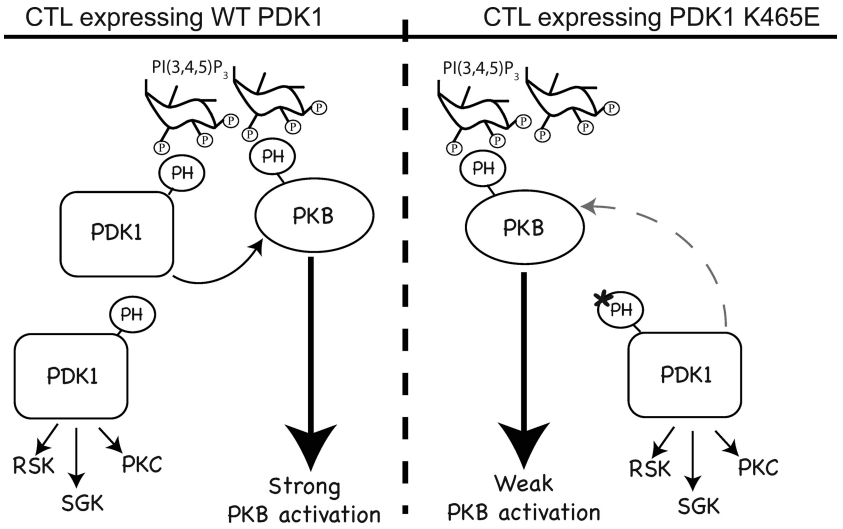

FIG. 7. Model of PDK1 signaling in WT and PDK1^{K465E} CTL. PDK1 is essential for the phosphorylation and activation of multiple AGC family kinases, such as RSK, SGK, PKCs, and PKB. PI(3,4,5)P_3 binding to PDK1 is never required for the PDK1-mediated phosphorylation of RSK, PKC, and SGKs, and the phosphorylation of these PDK1 substrates is not directly dependent on the production of PI(3,4,5)P3. PKB activation does require PI(3,4,5)P3 binding to the PKB PH domain. However, PI(3,4,5)P3 binding to the PDK1 PH domain is not essential for PKB activation, although it increases the efficiency of PKB phosphorylation by PDK1 by colocalizing the enzymes. In cells expressing the PDK1 K465E mutant, there is normal PDK1 catalytic activity and phosphorylation of PDK1 substrates such as RSK, PKCs and SGKs but only a weak suboptimal phosphorylation of PKB.

tor, KLF2, which is expressed at high levels in naive T cells (3, 10, 44). The expression of KLF2 is controlled by Foxo family transcription factors, and the PKB-mediated phosphorylation and inactivation of Foxo family members that occurs in immune-stimulated T cells results in the loss of KLF2 expression (16, 27). The present data show that PDK1$^{K465E/K465E}$ effector T cells do not downregulate the expression of KLF2 and retain the expression of CD62L, CCR7, and $S1P_1$ and retain the ability to home to secondary lymphoid tissue. The migratory pattern of effector T cells thus is controlled by PI(3,4,5)P_3 binding to PDK1, which supports the strong activation of PKB and phosphorylation and the inactivation of Foxo family transcription factors.

The observation that the strength of PKB activation dictates the pattern of chemokine receptors and adhesion molecules expressed on immune-activated T cells offers insight as to how signal strength can influence T-lymphocyte migration and determine the outcome of an immune response. In this context, there is an increasing recognition that the quality and quantity of T-cell immune responses are determined by the initial strength of antigen receptor triggering. In particular, the strength of TCR ligation can determine the kinetics of T-cell migration from secondary lymphoid tissues into the blood (51). The present data provide a molecular basis for this phenomenon. For example, high levels of PKB activity are required to downregulate the expression of KLF2 and its target gene *$S1P_1$*; the latter is the chemokine receptor that mediates T-cell exit from secondary lymphoid organs to the lymphatics (43). The downregulation of $S1P_1$ following immune activation is one of the mechanisms that retains activated T cells in lymph nodes and thus might occur only if there was a strong activation of PKB. A low-affinity TCR ligand that induced the weak activation of PKB thus might support T-cell survival and proliferation but would be unable to switch off $S1P_1$ expression and, hence, would be unable to retain cells in the secondary lymphoid tissue. The premature exit of activated T cells into the blood would curtail their exposure to antigen-primed antigen-presenting cells and cause an attenuated immune response.

ACKNOWLEDGMENTS

This project was supported by a Wellcome Trust Principal Research Fellowship (D.A.C.) and Program Grant no. 065975/Z/01/A.

We thank Elizabeth Farrell of the College of Life Sciences Cloning Service, University of Dundee for cloning of viral vectors; members of the Biological Services Resource Unit for mouse care; and members of the Cantrell laboratory for the critical reading of the manuscript.

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